NON-PROTEIN NITROGENOUS CONSTITUENTS OF FEEDING STUFFS

BY

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A. B. University of Illinois, 1915.

THESIS

Submitted in Partial Fulfillment of the Requirements for the

Degree of

MASTER OF SCIENCE

IN CHEMISTRY

IN

THE GRADUATE SCHOOL

OF THE

UNIVERSITY OF ILLINOIS

1918

UNIVERSITY OF ILLINOIS

THE GRADUATE SCHOOL

May 25, 1918

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Henry Charles Eckstein

ENTITLED Non-protein Nitrogenous Constituents of Feeding Stuffs

BE ACCEPTED AS FULFILLING THIS PART OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science in Chemistry

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PART I

METHOD USED TO DETERMINE NON-PROTEIN NITROGEN CONSTITUENTS.

Earlier Methods.

In reviewing the literature on determinations of non-protein nitrogen it is evident that there are many different methods for this group of nitrogenous constituents. With few exceptions, every precipitant of the protein molecule has been used for the separation of protein and non-protein nitrogenous substances. Among the older investigations, constant reference is made to the Stutzer method which in the main consists of precipitating the proteins with a suspension of cupric hydroxide. Attention was first called to the use of this reagent by Ritthausen[1], and a few years later Stutzer[2] published his first paper. The Stutzer method has often been severely criticised, but nevertheless it was only recently that methods were devised that can displace it. As pointed out by Schjerning,[3] the Stutzer reagent as well as many others either failed to completely precipitate the proteins or precipitated nitrogenous compounds that were unquestionably non-protein in nature. The particular difficulty with the Stutzer reagent was that it precipitated some of the acid-amide nitrogen and allowed some of the peptones to go into the filtrate. The work of Schjerning was very conclusive, and it is therefore quite evident that the older methods while they give results which may be fairly suitable for comparative work they cannot be used when a clean cut separation of protein and non-protein is desired.

More Recent Methods.

PRECIPITATION WITH ETHYL ALCOHOL. Among the more recent methods, that of precipitating with five volumes of 95 percent alcohol has proven to be unsatisfactory, because the alcohol not only precipitates some of the non-protein fraction but is also a good solvent for fats and thus gives filtrates, which

due to the large amounts of carbonaceous matter they contain, make the subsequent Kjeldahl nitrogen digestion without loss of ammonium sulfate a difficult process.

PRECIPITATION WITH METHYL ALCOHOL AND ZINC CHLORIDE. Folin[4] used in place of the ethyl alcohol a solution of methyl alcohol and at one time reported that when such precipitation was followed by the addition of a solution of zinc chloride in ethyl alcohol the filtrate contained only the non-protein nitrogen. This method was soon universally adopted in the study of this class of nitrogenous constituents, but was later discarded even by Folin himself, because as in the case of ethyl alcohol the precipitation was not free from possible error. Some nitrogenous lipoids are soluble in the alcohol, and further some other nitrogenous materials like the amino-acids, creatine, etc., which should remain in solution are precipitated. Finally attention is called to the same danger noted above in the discussion of precipitation with ethyl alcohol that the large amounts of carbonaceous matter extracted interfere with the subsequent total nitrogen determination.

PRECIPITATION WITH META-PHOSPHORIC ACID. The criticism of the precipitation with methyl alcohol and zinc chloride was made in the introductory remarks of a paper by Folin[5] and Dennis wherein they revived the use of metaphosphoric acid for the separation of protein from non-protein nitrogen. They found that in view of the fact that the method was quick, gave clear filtrates, and did not precipitate any of the non-protein nitrogen or did not allow any of the proteins to go through into the filtrate that metaphosphoric acid was ideal for the desired separation. Thus far the method has been applied only to blood. Experiments carried out by the writer showed that for partially hydrolyzed plant extracts, precipitation is rapid and the solutions obtained filter rapidly and give water clear filtrates. On the other hand, however, it has also been shown that for certain extracts of animal tissue filtration is extremely diffi-

cult and at times impossible.

PRECIPITATION WITH TRICHLORACETIC ACID. The method of Folin and Dennis was preceded somewhat by that of Greenwald[6] who in a preliminary paper reported that a good separation of protein and non-protein nitrogen could be obtained with the use of a two percent solution of trichloracetic acid. At times, however, the filtrates were not absolutely clear, but could be made so by the use of kaolin. Folin objected to the use of this method because of the two filtrations which are necessary when kaolin is added. In a second paper, Greenwald[7] overcame this difficulty by the use of a somewhat more concentrated trichloracetic acid solution and states that with the use of a five percent solution of the reagent he could avoid the addition of kaolin and likewise a second filtration. He also has shown that such filtrates are free from proteins, for upon adding picric acid or potassium mercuric iodide no precipitates are obtained. Furthermore Greenwald has proven that when a known amount of hydrolyzed protein is added to blood it can be quantitatively recovered in the filtrate. Another favorable point is that Greenwald has shown that the nitrogen in the filtrates after precipitation with trichloracetic acid check very well with those obtained by precipitating with mercuric chloride as recommended by Gettler and Baker.[8] Greenwald rightly concludes that trichloracetic acid gives a clean cut separation of protein and non-protein nitrogen. There is, nevertheless, one point that prevents the use of it for extracts studied in this investigation. Trichloracetic acid interferes with the amino-acid determination by the Van Slyke nitrous acid method, and it must therefore be removed before such an analysis can be made. This can be readily done by making the solution alkaline and boiling. As a result of this, the acid is broken down to carbon dioxide and chloroform, and in view of the fact that in investigations recently carried out it[9] seemed that chloroform gave rise to an increase of humin nitrogen in the hydrolysis of proteins this formation of chloroform seemed undesirable. This question is

being further studied in work analogous to that reported here.

PRECIPITATION BY BOILING SLIGHTLY ACIDIFIED EXTRACTS. Hart and Bentley[10] in investigation similar to those reported in this paper slightly acidify the hot water extracts of mature and immature plants with acetic acid and precipitate the soluble proteins by boiling the solutions for a short time.

PRECIPITATION WITH COLLOIDAL FERRIC HYDROXIDE. In a paper on the analysis of milk, Hill[11] removes the soluble protein by means of colloidal ferric hydroxide, and in a similar manner Van Slyke[12] and coworkers separate the protein and non-protein nitrogen of the blood. Van Slyke says, "In experiments carried out on Witte peptone and partially digested protein to be published later we have found furthermore that colloidal ferric hydrate not only lets all the amino-acids go thru into the filtrate, but that it also precipitates none of the intermediary products up to the albumoses, and none of these except some of complexity but little below that of the original proteins. As the precipitation of the native proteins is complete, colloidal ferric hydrate appears especially well adopted to our purpose."

DISCUSSION OF PRELIMINARY WORK THAT LED TO THE ADOPTION OF THE FINAL METHOD.

CHOICE OF PRECIPITANT. From a review of the literature on precipitants applicable for the separation of protein from non-protein nitrogen, it was quite evident that there were really only four reagents that could be used for the adequate separation of these constituents in feedingstuffs. These were trichloracetic acid, metaphosphoric acid, mercuric chloride, and colloidal ferric hydrate. Among these four, the last one seemed most suitable, because, as it developed in a preliminary experiment, extracts of feedingstuffs could be precipitated rapidly and the resulting solutions filtered to give water clear filtrates. Trichloracetic acid did not seem desirable at that time, because the subsequent formation of chloroform might lead to poor results in the peptide

nitrogen determination. The other methods were not considered, but it might be well to again call attention to the fact that with certain extracts, solutions precipitated with metaphosphoric acid are extremely hard to filter. As far as could be seen in the preliminary work, colloidal iron was an ideal precipitant and since Van Slyke had shown that the separation of protein and non-protein nitrogen was successful, the reagent was adopted for the separation of the extracts studied in this investigation.

EXTRACTION OF THE SAMPLES. The choice of a suitable method of extraction was probably the most difficult point to be settled in the perfection of the method. An extraction with cold nitrogen-free water was very desirable, but owing to the great difficulty encountered in the filtration of some of the samples, the following method of extraction was tentatively adopted. The samples were first extracted with small portions of absolute alcohol, then with similar amounts of 75 percent alcohol, then with 50 percent alcohol, then with 25 percent of alcohol, and finally with nitrogen-free water. This preliminary treatment with alcohol eliminated the substances responsible for the slow filtration noted above, and the subsequent extraction with water proceeded with no trouble. But in spite of this the method was not satisfactory, because of the alcohol introduced and in the course of some more detailed attempts to apply water extraction direct, the use of silk filters in four-inch Büchner funnels was suggested. With this modification, extraction was as rapid as with the preliminary treatment with alcohol, and extractions could be completed in the course of three hours. That such extraction was really complete was demonstrated by first extracting the feedingstuff with two and one half liters of water and then testing for completeness of extraction with an additional liter of water. The results of these tests show that the nitrogen not precipitated by colloidal iron in this second extract was reduced to less than 0.6 percent in the case of the alfalfa

hay; to less than 0.5 percent in the timothy; to less than 0.3 percent in the corn; and to less than 0.07 percent in the case of the blood meal.

DETERMINATION OF FREE AMMONIA. The Van Slyke[13] method of determining ammonia by distillation in vacuo of solutions made alkaline with a suspension of 10 percent calcium hydroxide was most suitable for the determination of this form of nitrogen in plant extracts. In a preliminary outline of the method, this analysis was to be made upon the filtrate from the colloidal iron, but as an added precaution this was checked by a determination of ammonia in the original water extract. In the course of quite a few determinations, it was invariably found that the filtrates gave higher results than the original extracts. Total nitrogen determination on the reagents showed that the increase was not due to these, and the logical conclusion was to suppose that during the short boiling to affect precipitation, hydrolysis of the simpler amides took place. For this reason, all free ammonia determinations were made upon the original water extracts.

DETERMINATION OF THE FREE AMINO-ACID NITROGEN. In the original presentation of the Van Slyke nitrous acid method for amino-acid nitrogen,[13] it was pointed out that ammonia interferes with the determination, and consequently this substance had to be removed from the non-protein fraction before such analyses could be made. This was accomplished by distilling in vacuo as per the Van Slyke method and determining the amino-acids in the residual solution. To do this the calcium hydroxide was neutralized with hydrochloric acid and then concentrated to a very small volume. The resulting amino-acid determinations were however not satisfactory, since marked differences were noted between the determinations upon duplicate extracts of the same feedingstuff. A consideration of all factors involved limited the difficulty to the use of hydrochloric acid in the neutralization of the calcium hydroxide. No marked excess was of course used, but, since the solutions were finally concentrated to a volume as small as 15 cc., the presence of only a slight excess in the original 500 cc. volume

would be quite pronounced in the final concentrated solution. It was thought that this difficulty might be overcome by the use of a weaker acid for neutralization and this assumption proved to be true, for when acetic acid was substituted for the hydrochloric the subsequent amino-acid determinations checked very well. The discrepancies in the previous amino-acid determination were therefore explained on the basis of hydrolysis of peptide linkings by the strong acid.

DETERMINATION OF THE FREE AND COMBINED ACID-AMIDE NITROGEN. This form of nitrogen results from the hydrolysis of free and combined acid-amide linkings. The important point to settle in connection with this determination was the minimum time required for the complete hydrolysis of these forms occurring in the plant extracts. These constituents are determined by Van Slyke[13] in his method for protein analysis by neutralizing the completely hydrolyzed protein mixture with calcium hydroxide and distilling in vacuo. This gave a method for analysis as well as one that could be used to determine when the amides were completely hydrolyzed. With this in mind, fractions of the filtrate from the colloidal iron were treated with such an amount of concentrated hydrochloric acid to make a final concentration of 20 percent acid and boiled for periods of 6, 8, 12 and 16 hours. The analyses of these solutions showed that, whereas no increase took place after the 12th hour, sufficient change did take place between the 8th and 12th hours to warrant a hydrolysis of 12 hours in all subsequent determinations.

DETERMINATION OF THE COMBINED AMINO-ACID NITROGEN. Besides hydrolyzing all of the acid-amides present in the filtrate from the colloidal iron, any peptides which escape precipitation will be broken down to amino-acids during the treatment with the 20 percent hydrochloric acid discussed in the preceding paragraph. These peptides can be then determined by neutralizing the solution after determining the free and combined acid-amide nitrogen, and concentrating as in the free amino-acid determination.

The Method Finally Adopted.

The method as finally adopted from the experiments discussed in the preceding paragraphs is briefly stated in the following:

EXTRACTION. Ten to twenty-five grams of the feedingstuff, placed upon a four-inch Büchner funnel, were washed repeatedly with cold water until the filtrate measured 2400 cc. The extract was diluted to 2500 cc., and the following determination made:

(a) THE TOTAL SOLUBLE NITROGEN was determined in 200 cc. portions of the extract in triplicate.

(b) THE FREE AMMONIA was determined in a 500 cc. portion by the Van Slyke vacuum method.

PRECIPITATION OF THE NATIVE PROTEINS. The native proteins were separated in a 1250 cc. portion of the extract by heating to boiling and adding, drop by drop, while constantly stirring 7.5 cc. of colloidal ferric hydroxide (containing five percent of Fe_2O_3). The solution was then boiled for one minute, and one cc. of a solution of crystallized magnesium sulfate (made by dissolving $MgSO_4.7H_2O$ in an equal weight of water), was added to coagulate the excess of colloidal ferric hydroxide. The solution was again boiled for one minute. After standing over night, the solution was filtered through folded filters, and the precipitate thoroughly washed with hot water. The filtrate and washings after being diluted to 1500 cc. were used for the following determinations:

(c) THE TOTAL NITROGEN not precipitated by colloidal ferric hydroxide was determined in duplicate in 200 cc. portions.

(d) THE FREE AMMONIA was determined in one portion of 500 cc. by the Van Slyke vacuum method.

(e) THE FREE AMINO-ACID NITROGEN was determined by slightly acidifying the residual mixture left in the distilling flask from the above determination

in (d) with acetic acid, filtering and washing. The filtrate and washings were concentrated on the water bath to about 15 cc., and the concentrated solution washed into a 25 cc. measuring flask and diluted to the mark. The amino-acid nitrogen was determined in duplicate by the Van Slyke nitrous acid method.

(f) THE FREE AND COMBINED ACID-AMIDE NITROGEN was determined in a 500 cc. portion of the solution by adding enough of concentrated hydrochloric acid to give a concentration of 20 percent, and then boiling under a reflux condenser for 12 hours. The excess of hydrochloric acid was removed by evaporation in vacuo, and the ammonia determined by the Van Slyke vacuum method. One-half of the ammonia determined above in (b) subtracted from the weight of the ammonia nitrogen obtained in this determination gave the weight of free and combined acid-amide nitrogen in one-half the quantity of the feedingstuff originally taken for analysis.

(g) THE HUMIN NITROGEN, which was formed in the above determination by boiling the aqueous extract of the feedingstuff with 20 percent hydrochloric acid, was filtered off and washed as per the Van Slyke method. The nitrogen in the humin precipitate was determined by the Kjeldahl method.

(h) THE COMBINED AMINO-ACID NITROGEN was determined by slightly acidifying the filtrate and washings from the above determination in (g) and then evaporating the resulting solution on the steam bath to a volume of about 30 cc. The concentrated solution was washed into a 50 cc. measuring flask, made up to the mark, and the amino-acid nitrogen determined in duplicate in 10 cc. portions of this solution by the Van Slyke nitrous acid method. The free amino-acid nitrogen determined above in (e) subtracted from the free and combined amino-acid nitrogen.

(i) THE RESIDUAL SOLUBLE NITROGEN, which did not respond to any of the above tests, was obtained by subtracting the sum of one half of the free ammonia obtained in (b), the free amino-acid obtained in (e), the free and combined

acid-amide nitrogen obtained in (f), the humin nitrogen obtained in (g), and the combined amino-acid nitrogen obtained in (h) from the total nitrogen obtained in (c).

PART II

APPLICATION OF THE METHOD TO THE DETERMINATION OF THE NON-PROTEIN NITROGENOUS CONSTITUENTS OF FEEDINGSTUFFS.

THE OBJECT of this part of the investigation was first to demonstrate that the non-protein nitrogen of these feeds would not interfere with the application of the Van Slyke method for the determination of the chemical groups characteristic of the different amino-acids of proteins, and second to obtain a fairly complete picture of the different forms of the non-protein nitrogen.

THE CHARACTER OF THE RESULTS OBTAINED is apparent from the data given in Tables 1, 2, and 3. In Table 1, the non-protein nitrogenous constituents of feedingstuffs are given expressed in the total nitrogen of the feedingstuff. In Table 2, the non-protein nitrogenous constituents are expressed in percent of the feedingstuffs. In Table 3, the non-protein nitrogenous constituents are expressed in percent of the total soluble nitrogen not precipitated by colloidal ferric hydroxide.

TABLE 1. THE NONPROTEIN NITROGENOUS CONSTITUENTS OF FEEDINGSTUFFS.

Results Expressed in Percent of the Total Nitrogen of the Feedingstuffs.

	In the original extract.			In the filtrate from the colloidal iron precipitate.					
Feed.	Total nitrogen.	Free ammonia nitrogen.	Nitrogen precipitated by colloidal iron.	Total nitrogen.	Humin nitrogen.	Free amino-acid nitrogen.	Free and combined acid-amide nitrogen.	Combined amino-acid nitrogen.	Residual soluble nitrogen.
Alfalfa hay.....	28.58	1.07	11.07	17.51	1.40	5.08	3.40	3.10	3.46
Alfalfa hay.....	28.21	1.15	10.63	17.58	1.48	5.00	3.29	3.15	3.51
Average.....	28.40	1.11	10.85	17.55	1.44	5.04	3.35	3.13	3.49
Timothy hay.....	23.58	1.44	8.38	15.20	2.67	4.82	2.67	1.19	2.41
Timothy hay.....	23.92	1.49	8.27	15.65	2.70	4.84	2.60	1.14	2.88
Average.....	23.75	1.47	8.33	15.43	2.69	4.83	2.64	1.17	2.65
Blood meal.....	2.33	0.19	0.49	1.84	0.04	0.58	0.47	0.48	0.08
Blood meal.....	2.33	0.14	0.43	1.90	0.05	0.57	0.49	0.52	0.13
Average.....	2.33	0.17	0.46	1.87	0.05	0.58	0.48	0.50	0.11
Corn.....	7.17	0.66	1.55	5.62	0.24	2.16	1.04	0.57	0.95
Corn.....	7.74	0.70	2.06	5.68	0.36	2.17	1.08	0.51	0.86
Average.....	7.46	0.68	1.81	5.65	0.30	2.17	1.06	0.54	0.91
Clover hay.....	16.50	1.99	2.29	14.21	1.77	4.32	1.40	0.19	4.54
Clover hay.....	16.60	1.98	2.37	14.23	1.78	4.42	1.18	0.29	4.58
Average.....	16.55	1.99	2.33	14.22	1.78	4.37	1.29	0.24	4.56

TABLE 2. THE NONPROTEIN NITROGENOUS CONSTITUENTS OF FEEDINGSTUFFS.

Results Expressed in Percent of the Feedingstuffs.

	In the original extract.			In the filtrate from the colloidal iron precipitate.					
Feed.	Total nitrogen.	Free ammonia nitrogen.	Nitrogen precipitated by colloidal iron.	Total nitrogen.	Humin nitrogen.	Free amino-acid nitrogen.	Free and combined acid-amide nitrogen.	Combined amino-acid nitrogen.	Residual soluble nitrogen.
Alfalfa hay.....	0.751	0.028	0.290	0.461	0.037	0.134	0.089	0.081	0.092
Alfalfa hay.....	0.741	0.030	0.279	0.462	0.039	0.131	0.086	0.083	0.093
Average.....	0.746	0.029	0.285	0.462	0.038	0.133	0.088	0.082	0.093
Timothy hay.....	0.202	0.012	0.072	0.130	0.023	0.042	0.023	0.010	0.020
Timothy hay.....	0.205	0.013	0.071	0.134	0.023	0.042	0.022	0.008	0.026
Average.....	0.204	0.013	0.072	0.132	0.023	0.042	0.023	0.009	0.023
Blood meal.....	0.327	0.026	0.069	0.258	0.005	0.082	0.065	0.068	0.012
Blood meal.....	0.326	0.019	0.061	0.265	0.007	0.079	0.069	0.073	0.018
Average.....	0.327	0.023	0.065	0.262	0.006	0.081	0.067	0.071	0.015
Corn.....	0.103	0.009	0.022	0.081	0.004	0.031	0.015	0.008	0.014
Corn.....	0.111	0.010	0.029	0.082	0.005	0.031	0.015	0.006	0.015
Average.....	0.107	0.010	0.026	0.082	0.005	0.031	0.015	0.007	0.015
Clover hay.....	0.330	0.040	0.045	0.285	0.035	0.090	0.028	0.004	0.088
Clover hay.....	0.332	0.040	0.047	0.285	0.036	0.091	0.024	0.006	0.088
Average.....	0.331	0.040	0.046	0.285	0.036	0.091	0.026	0.005	0.088

TABLE 3. THE NONPROTEIN NITROGENOUS CONSTITUENTS OF FEEDINGSTUFFS.

Results Expressed in Percent of the Total Soluble Nitrogen not Precipitated by Colloidal Ferric Hydroxide.

Feed.	Total nitrogen.	Free ammonia nitrogen.	Humin nitrogen.	Free amino-acid nitrogen.	Free and combined acid-amide nitrogen.	Combined amino-acid nitrogen.	Residual soluble nitrogen.
Alfalfa hay..	100.00	6.32	8.21	28.72	19.09	17.83	19.89
Timothy hay..	100.00	9.53	17.43	31.30	17.11	7.58	17.07
Blood meal...	100.00	9.09	2.67	31.02	25.67	26.74	5.88
Corn.....	100.00	12.04	5.31	38.41	18.76	9.56	16.11
Clover hay...	100.00	13.99	12.52	30.73	9.07	1.69	32.07

It is apparent that this method of determining the non-protein nitrogenous constituents of feedingstuffs gives a fairly complete picture of the different forms of nitrogen represented in the so-called non-protein nitrogenous constituents.

It is also apparent that the non-protein nitrogenous constituents consist largely of the forms of nitrogen that result from the decomposition of proteins by hydrolysis. In other words, the sum of amide nitrogen, the humin nitrogen, the free amino-acid nitrogen, the combined amino-acid nitrogen and the free and combined acid-amide nitrogen represented in the non-protein nitrogenous constitutes from 80 percent in the case of the alfalfa hay to 94 percent in the case of the blood meal, of the non-protein nitrogen. Further, it is probable that at least 50 percent of the residual soluble nitrogen not precipitated by colloidal iron hydroxide represents the non-amino nitrogen present in the free and the combined amino-acids determined. It is impossible at present to tell definitely what the remaining 3 to 10 percent of the residual soluble nitrogen which does not correspond to any of the tests here applied, represents. However, it is quite evident that only a small part, if any, of the non-protein nitrogenous constituents of foods and feedingstuffs can interfere with the application of the Van Slyke method for the determination of the chemical groups characteristic of the different amino-acids of proteins to the estimation of the free and combined amino-acids

and amides of feedingstuffs.

It is also evident that the so-called "amide" nitrogen of feedingstuffs is composed largely of free amino-acid and peptide linkings. In Table 3 it is shown that the nitrogen in these latter forms including the humin nitrogen constitutes from 53 to 63 percent of the water soluble nitrogen not precipitated by colloidal ferric hydroxide.

The free and combined acid-amide nitrogen varied from 9.07 to 25.67 percent and the free ammonia from 6.23 to 13.99 percent.

It is interesting to note from the results compiled in Table 1 that there are marked differences between the three types of feedingstuffs represented therein. The total nitrogen not precipitated by colloidal ferric hydroxide ranged from 14.22 to 17.55 percent in the three hays, whereas it was only 5.55 percent in the case of the corn, and 1.87 percent in the blood meal. Marked differences are also found in the humin nitrogen determinations. Here again the three hays run very close together varying only from 1.44 to 2.69 percent. In the corn, however, only 0.30 percent is in the form of humin nitrogen and in the blood only 0.05 percent. Table 1 reveals the fact that there are also marked differences in the quantities of amino-acids present. Those present in the hays range from 4.37 to 5.04 percent, while those of the corn decrease to 2.17 percent and those in the blood meal to 0.58 percent. Similar results are noted in the residual soluble nitrogen. On the other hand, the table shows marked differences in the percent of combined amino-acid and free and combined acid-amide nitrogen.

COMPARISON OF THE METHOD AND THE RESULTS WITH THOSE OF HART AND BENTLEY.

Hart and Bentley have made an investigation very similar to that presented in this paper. Table 4 shows the results obtained by them. A study of the table shows that the methods do not check. Therefore, a detailed presentation of their method is given, because it is possible that the differences can be explained by a study of the methods.

TABLE 4. DISTRIBUTION OF WATER-SOLUBLE NITROGEN IN SOME COMMON PLANT MATERIALS. HART AND BENTLEY.

Feed.	Stages of Growth.	Total nitrogen.	In percent of total nitrogen					
			Water soluble.	Ammonia nitrogen.	Acid-amide nitrogen.	Amino-acid nitrogen.	Rest nitrogen.	Peptide nitrogen.
Alfalfa	In blossom	3.67	27.9	0.87	1.74	17.7	7.6	----
Red clover	In blossom	4.48	18.2	0.35	0.35	9.9	7.6	----
Timothy	Headed green	1.48	25.9	0.26	1.34	17.1	7.2	----
Clover hay	Mature	2.53	9.3	None	None	4.2	5.1	----
Alfalfa hay	Mature	2.28	23.50	None	7.30	10.0	6.2	----

Comparison of Methods.

In the following paragraphs a brief presentation of the method of Hart and Bentley is given.

METHOD OF EXTRACTION AND PRECIPITATION. Twenty-five grams of the air dried material were extracted with small portion of HOT water until nearly 500 cc. of filtrate were obtained. The filtrate was then slightly acidified with acetic acid, boiled for a few minutes, cooled, made up to 500 cc. and filtered.

DETERMINATION OF FREE AMMONIA. Twenty-five cc. were areated according to the method of Folin. Under these conditions no free acid-amide nitrogen was split off.

ESTIMATION OF ACID-AMIDE NITROGEN. Enough of concentrated hydrochloric acid was added to 25 cc. of the filtrate to make a concentration of 20 percent acid, boiled for 30 minutes, cooled, exactly neutralized and made up to 100 cc. The total ammonia determined in this solution by areation corrected for the free ammonia gave the acid-amide nitrogen.

DETERMINATION OF FREE AMINO-ACID NITROGEN. The amino-acid nitrogen was determined directly upon the solution by the Van Slyke nitrous acid method.

DETERMINATION OF PEPTIDE NITROGEN. The water extract was hydrolyzed for five hours with sulfuric acid, then neutralized, and analyzed for amino-acid nitrogen by the Van Slyke nitrous acid method.

In comparing the two methods attention should first be called to the fact that the fractions used for analysis by Hart and Bentley are relatively small when compared with those used in this investigation. In determining free ammonia and acid amide nitrogen, Hart and Bentley use 1-20 of their total extract as compared with 1-5 in the free ammonia and 1-6 in free and combined acid-amide nitrogen in the method as perfected in this report. Likewise, it can be seen that in the amino-acid determination one cc. in the burrette of the Van Slyke apparatus corresponds to 1-500 of the extract of Hart and Bentley as compared with 1-150 in the other method. These facts show that the source of error in the method developed in this investigation is much less than that devised by Hart and Bentley.

Comparison of Results.

THE FREE AND COMBINED ACID-AMIDE NITROGEN. A study of Tables 3 and 4 shows that the free and combined acid-amide nitrogen reported in this investigation varied from 9.07 to 25.67 percent whereas Hart and Bentley state that the "free acid-amide" nitrogen seldom exceeded 20 percent and was more often below 10 percent. These results are not directly comparable. It does seem, however, that the results reported in Table 4 do not represent merely the free acid-amide nitrogen, for it is a well-known fact that proteins and peptides very readily yield ammonia when boiled with 20 percent acid. But even if these methods are not directly comparable, it is really surprising that Hart and Bentley found no free acid-amide radicles in the extract of the clover hay whereas the writer reports as much as 9 percent as expressed in percent of the total non-protein nitrogen.

THE FREE AMMONIA. A further consideration of Tables 3 and 4 brings out the rather surprising point that while Hart and Bentley report not even so much as a trace of free ammonia in the non-protein nitrogen fraction of alfalfa and timothy hay, Table 3 shows 6.23 percent of the non-protein nitrogen of the

alfalfa in that form, and 13.99 percent of the non-protein nitrogen of the timothy hay. These discrepancies are unquestionably due to differences in the extraction of the original air dried samples, because it has been shown that when extractions are made with hot water as recommended by Hart and Bentley only a small trace of ammonia is obtained, but when this hot water is slightly acidified with hydrochloric acid (0.185 percent) the resulting extract contained 4.44 percent of its total soluble nitrogen, not precipitated by colloidal iron as free ammonia. A water extract of alfalfa hay is distinctly alkaline to litmus paper.

THE FREE AND COMBINED AMINO-ACID NITROGEN. As a whole the results obtained on this group of the constituents check quite well. The nitrogen in these forms including the humin nitrogen range from 53 to 63 percent of the water soluble nitrogen not precipitated by the colloidal iron. Hart and Bentley reported 50 to 70 percent of the water soluble nitrogen of immature and mature plants, in the same form. Nevertheless, it must be pointed out that in the latter determinations the results should be too high, because, as shown in the first paper on the determination of amino-acids by nitrous acid, ammonia when present gives high results. In discussing the details of the method perfected in this investigation, it was shown that the ammonia was invariably removed before all amino-acid determinations, and in view of this the results obtained should be lower and unquestionably more accurate than those reported by Hart and Bentley who do not remove the ammonia.

PART III

CHANGES IN THE COMPOSITION OF ALFALFA HAY AT DIFFERENT STAGES OF GROWTH.

INTRODUCTION.

A study of the non-protein nitrogenous constituents of plants at different stages of growth is important, because the results obtained may give some insight on the complex problem of protein synthesis in plants. Much work has already been done on this problem, but as yet nothing definite can be concluded

regarding the synthesis of these nitrogenous complexes. A review of the literature shows that this problem has been attacked in several ways.

In the first place, a group of investigators attempted to solve the problem by limiting the nutrients to mixtures of known composition, and tracing the disappearance of the more simple compounds such as nitrates and the ammonium salts and the appearance of the protein complexes. Maze,[14] for example, demonstrated that a decrease of ammonium salts was accompanied by an increase in proteins, and Zaleski[15] showed that when leaves were placed in solutions containing sucrose and nitrates that the concentration of the nitrates decreased, while the proteins in the leaves increased. On the other hand, controls in sucrose and water showed no such increase.

In the second place, another group of chemists sought evidence to explain this process by analyzing plants at different stages of growth. Much work[16] has been done on the APPROXIMATE analysis of plants at different stages of growth, and, altho nothing conclusive as to protein synthesis in plants can be gained from such work, it is, nevertheless, important, because it shows that marked changes do occur in all of the constituents of the plant as it matures. The Utah Experiment Station[17] has devoted much time to the study of alfalfa hay at different stages of growth, and has supplemented the approximate analyses with albuminoid and so-called "amide" nitrogen determinations. They find that a decrease of crude ash, fat, moisture, and "amide" nitrogen takes place as the plant matures, and, altho nothing can be derived from such evidence to explain the mechanism of protein synthesis, it is, nevertheless, important to note that similar changes took place in the constituents obtained in the approximate analysis of the alfalfa hay studied in this investigation.

The more EXACT analyses of the nitrogenous constituents of plants at different stages of growth, tho they do not by any means clear up this complex problem, do, however, give some insight as to the character of the changes that

may be taking place.

Jost,[18] for example, studied the relation of asparagine to protein synthesis, and found that asparagine increased at the expense of the amino-acids. He concludes that asparagine is an intermediate product between the amino-acids and the proteins. Borodin[19] states, in this connection, that under normal conditions asparagine is employed in the formation of proteins as quickly as it is formed, and, while it can be detected if the plants are grown in the dark, it is rarely found in amounts sufficient for detection during normal vegetation. While nothing can be said of the mechanism of asparagine formation, it has been suggested that it can arise from a combination of ammonia with aspartic acid to give ammonium aspartate which by loss of a molecule of water gives the desired asparagine. The evidence produced to show that asparagine is an intermediate product between the amino-acids and the proteins is not very conclusive, and the opinion now generally accepted is that asparagine is a secondary product, because, tho it appears in large amounts, it is found in greater abundance in the developing parts than in those where the proteins are stored.

Kellner[20] is of the belief that the amino-acids are the first stage of nitrogen assimilation, and Zaleski[21] found that during the ripening of the pea seed an increase of proteins took place at the expense of the amino-acids and the organic bases. He also states that this action was hastened by desiccation and concluded that protein synthesis was of the nature of a condensation. The results were not so well marked with all seeds; thus under similar conditions no increase took place with maize, while with the sunflower seed there was even evidence of protein decomposition.

Boudisch[22] also believes that protein synthesis is a condensation process. Furthermore, he is of the opinion that photochemical action is a very important factor. In studies on the effect of light on nitrates and nitrites, he comes to the conclusion that these are first reduced and the ammonia which is

formed is oxidized either by oxidases or ultraviolet light. The resulting product then condenses with formaldehyde to form aci-nitro methane which is very reactive, and which is shown to be capable of forming several complexes frequently found in plants. The idea of the importance of photochemical reactions in protein synthesis may be criticised, because it has been shown that the synthesis can take place in the dark and in tissues free of chlorphyll, but, nevertheless, protein synthesis depends upon an adequate supply of carbohydrates and so eventually upon photosynthesis.

In addition to these views, mention should be made of the work of Treub[23] who holds that hydrocyanic acid is an important factor in protein synthesis. He concludes from his investigations that this acid is the first recognizable product of nitrogen assimilations and possibly the first organic nitrogen compound formed. His evidence is, however, not conclusive and his theory not well accepted.

This discussion of protein synthesis includes a brief summary of this complex subject. It is apparent that the present knowledge of this question is incomplete. All that is agreed upon is that the leaves are important centers of protein synthesis, that nitrates are elementary substances, and that these are reduced to nitrites. Beyond this point, however, nothing definite can be said.

RESULTS OBTAINED IN THIS INVESTIGATION.

The study of the alfalfa hay at different stages of growth in this investigation was made upon samples taken weekly from the time the hay was 6 inches high until 5 weeks after cutting time. Table 5 gives a summary of the stages of growth, the mean humidity, the total rainfall, and the average mean daily temperature of the ten periods. The analyses are divided into two distinct groups. First, the APPROXIMATE analysis of the constituents of the plant, and second, the analysis of the non-protein nitrogenous constituents.

APPROXIMATE ANALYSIS OF THE ALFALFA HAY AT DIFFERENT STAGES OF GROWTH.

The approximate chemical composition of the fresh substance of the samples of alfalfa are given in Table 6. It will be plainly noted that the dry substance gradually increased from June 28, when it was 14.4 percent, to August 30, when the dry substance was 36.6 percent. It is also very evident that the nitrogen-free extract increased at first very rapidly from June 28, when it was 4.76 percent, to July 26 when it was 16.30 percent. From this time on, there was somewhat of a decrease in the nitrogen-free extract until near the end, when the samples taken August 23 and 30 showed increases.

The crude protein in the several samples of the fresh substance of the alfalfa showed no general and continuous variations; but the percentages of crude protein on this basis fluctuated within comparatively wide limits. It is apparent that the ether extract gradually increased from the first sample collected June 28 to the fifth sample collected July 26; and from this time on there were slight decreases with irregular fluctuations.

In general, there was an increase in the percentage of crude ash in the fresh substance of the alfalfa from 1.41 for the sample collected June 28 to 2.25 for the sample collected August 30. It will be noted that there was a continuous and marked increase in the percentage of crude fiber from the first sample collected June 28 to the seventh sample collected August 9. The former being 2.0 percent and the latter 10.75 percent. The sample collected August 16 showed a slight decrease in the percentage of crude fiber and the two samples following, again showed increases. The same remarks, of course, that were made as to the crude protein apply also to the total nitrogen.

The approximate chemical composition of the water-free substance of the alfalfa samples are given in Table 7. It will be noted in the first place that there was a marked increase even upon this basis in the nitrogen-free ex-

tract from June 28 to July 26. From this time on it decreased for two weeks; then followed a slight increase for two weeks with a final slight drop in the percentage of nitrogen free extract.

TABLE 5. DATES OF COLLECTION OF THE SAMPLES OF THE SECOND GROWTH OF ALFALFA, AND WEATHER CONDITIONS.

Date collected.	Days of growth after first cutting.[1]	Total rainfall for week preceding date of collection.	Average mean daily temperature for week preceding date of collection.	Average mean daily humidity for week preceding date of collection.	Remarks.
1916					
June 28	12	1.11	70.9	72.9	Rank growth. 4 to 6 inches.
July 5	19	0.07	79.0	67.6	Growth of 8 to 10 inches.
July 13	27	0.20	79.1	65.4	Growth of 12 to 15 inches. 5 percent in bloom.
July 19	33	Trace	86.1	65.7	Growth of 18 to 20 inches. Much in bloom.
July 26	40	0.20	83.6	55.5	Growth of about 20 inches. In full bloom.
Aug. 2	47	0.36	85.1	57.4	Some dead leaves. 3rd growth 2 to 3 inches.
Aug. 9	54	0.07	84.4	64.5	Wholly unpalatable to hogs. 3rd growth about 8 inches.
Aug. 16	61	1.27	75.8	74.7	Very woody.
Aug. 23	68	Trace	82.0	64.3	Very woody. Lost many leaves.
Aug. 30	75	0.02	71.3	57.0	Many leaves lost.

[1] The first cutting was made June 16, 1916, and the second July 22, 1916.

TABLE 6. ANALYSIS OF SECOND GROWTH ALFALFA.

Results expressed on the basis of the fresh substance.

Date collected. 1916	Dry substance.	Nitrogen free extract.	Crude protein Nx6.25.	Ether extract.	Crude ash.	Crude fiber.	Total nitrogen.
June 28	14.41	4.76	5.57	0.60	1.41	2.07	0.891
July 5	20.34	7.84	6.11	0.83	1.61	3.95	0.978
" 13	22.24	8.85	5.23	0.82	1.50	5.84	0.836
" 19	24.80	10.56	5.07	0.85	1.57	6.75	0.812
" 26	35.19	16.30	6.03	1.22	1.92	9.71	0.965
Aug. 2	34.37	15.40	5.78	1.03	1.92	10.24	0.925
" 9	33.64	14.42	5.47	1.01	1.99	10.75	0.875
" 16	32.91	14.23	5.50	0.94	1.88	10.36	0.880
" 23	35.63	15.55	5.75	1.03	2.13	11.17	0.920
" 30	36.56	15.60	5.79	1.09	2.25	11.82	0.926

TABLE 7. ANALYSIS OF SECOND GROWTH ALFALFA.

Results expressed on the basis of the water-free substance.

Date collected 1916	Nitrogen free extract.	Crude protein Nx6.25.	Ether extract.	Crude ash.	Crude fiber.	Total nitrogen.
June 28	33.04	38.04	4.18	9.77	14.38	6.183
July 5	38.55	30.05	4.07	7.90	19.42	4.808
" 13	39.79	23.51	3.70	6.75	26.25	3.761
" 19	42.58	20.46	3.43	6.31	27.23	3.272
" 26	46.34	17.14	3.46	5.46	27.61	2.742
Aug. 2	44.81	16.81	3.00	5.60	29.79	2.691
" 9	42.87	16.26	3.00	5.92	31.97	2.600
" 16	43.25	16.72	2.85	5.72	31.47	2.674
" 23	43.65	16.14	2.88	5.98	31.34	2.582
" 30	42.68	15.83	2.98	6.16	32.34	2.533

The percentage of crude protein upon the water-free substance of the alfalfa was remarkably high, namely, 38 percent in the first sample collected June 28. From this time on the percentage of crude protein rapidly dropped until July 26; then there was a further slight decrease in the percentage of crude protein until August 9. Then the following week there was a slight rise and in the last two weeks there was again a slight decrease. There was from the beginning to the end a slight but almost continuous decrease in the percentage of ether extract in the water-free substance of the alfalfa. On this same basis it is also apparent that there was a considerable decrease in the percentage of crude ash. In the first samples of young alfalfa collected the percentage of crude ash in the first sample collected June 28 amounted to 9.77 percent while the percentage in the fifth sample collected July 27 had fallen to 5.46. From this time on there was a continuous slight increase in the percentage of ash in the alfalfa excepting in the case of the sample collected August 16 when there was a slight decrease from the percentage shown the previous week.

There was a very marked increase in the percentage of crude fiber in the second and third samples and increases in the samples collected July 19 and 26; and still further increase in the sample collected August 2 and 9. In the sample collected August 16 and 23, there were slight decreases with a final increase in the last sample collected August 30. The percentage of crude fiber in the first sample equaled 14.38 percent while in the last sample, it amounted to 32.34 percent.

NON-PROTEIN NITROGENOUS CONSTITUENTS OF ALFALFA HAY AT DIFFERENT STAGES OF GROWTH.

DISCUSSION OF RESULTS OBTAINED.

The character of the results obtained is apparent from the data given in Tables 8 and 9. In Table 8, the non-protein nitrogenous constituents are expressed in percent of the total nitrogen. In Table 9, the constituents are expressed in percent of the water free hay.

TABLE 8. FORMS OF NON-PROTEIN NITROGEN IN ALFALFA CUT AT DIFFERENT STAGES OF GROWTH.
Results expressed in percent of the total nitrogen of the alfalfa.

Date 1916	Total nitrogen in					Nitrogen in the filtrate from the colloidal iron in the form of							
	Ether extract.	Water extract.	Residue insoluble in ether and water.	Precipitate produced by the colloidal iron.	Protein nitrogen.	Total.	Ammonia.	Humin.	Free and combined acid-amide.	Free amino.	Combined amino.	Free and combined amino.	Residual soluble nitrogen in the filtrate.
June 28	0.837	36.670	62.527	1.362	63.889	35.308	4.443	1.945	2.401	10.079	6.100	16.179	10.316
" "	0.829	36.241	62.924	0.943	63.867	35.298	4.153	1.860	2.392	10.191	5.942	16.133	10.794
Average	0.833	36.456	62.726	1.153	63.875	35.303	4.298	1.903	2.397	10.135	6.021	16.156	10.555
July 5	0.669	39.694	59.637	2.021	61.658	37.672	3.281	2.107	4.277	11.566	7.884	19.450	8.557
" "	0.636	39.765	59.561	2.110	61.671	37.654	3.230	1.385	4.745	11.693	7.740	19.433	8.901
Average	0.653	39.730	59.599	2.066	61.665	37.663	3.256	1.746	4.511	11.630	7.812	19.441	8.729
July 13	0.534	37.367	62.099	1.653	63.752	35.714	2.908	2.053	4.869	12.091	5.903	18.327	7.419
" "	0.557	37.610	61.833	2.015	63.848	35.595	2.887	2.079	5.197	12.117	5.944	18.062	8.042
Average	0.546	37.489	61.966	1.834	63.800	35.655	2.898	2.066	5.033	12.104	5.924	18.195	7.731
July 19	0.504	39.853	59.646	4.382	64.028	35.471	2.670	2.305	6.675	13.502	3.649	17.151	6.652
" "	0.490	39.745	59.766	3.930	63.696	35.814	2.656	2.109	5.236	13.537	3.647	17.184	8.630
Average	0.497	39.799	59.706	4.156	63.862	35.643	2.663	2.207	5.956	13.520	3.648	17.168	7.641
July 26	0.457	36.288	63.255	4.272	67.527	32.016	2.642	2.494	5.632	12.120	5.046	17.195	4.064
" "	0.476	36.475	63.178	4.360	67.538	32.114	2.380	2.462	5.700	12.067	5.075	17.113	4.496
Average	0.467	36.382	63.217	4.316	67.532	32.065	2.511	2.478	5.666	12.094	5.061	17.154	4.280
August 2	0.425	37.385	62.191	5.559	67.750	31.826	1.723	2.260	6.717	11.637	4.822	16.459	4.668
" "	0.406	37.327	62.266	5.489	67.755	31.839	1.699	2.184	6.839	11.697	4.802	16.483	4.630
Average	0.416	37.356	62.229	5.524	67.750	31.834	1.711	2.222	6.778	11.667	4.804	16.471	4.654
August 9	0.328	35.235	64.436	5.740	70.176	29.495	1.209	2.039	6.166	12.974	3.137	16.111	3.970
" "	0.296	35.256	64.449	5.519	69.968	29.735	1.169	2.087	6.217	12.969	2.764	15.732	4.531
Average	0.312	35.246	64.443	5.630	70.072	29.616	1.189	2.063	6.192	12.972	2.951	15.922	4.251
August 16	0.346	31.334	68.320	6.024	74.344	25.309	1.060	1.259	4.887	11.371	1.870	12.908	4.862
" "	0.441	31.551	68.008	6.281	74.289	25.270	1.142	1.913	4.592	11.355	1.853	13.208	4.414
Average	0.394	31.443	68.164	6.153	74.317	25.290	1.101	1.586	4.740	11.363	1.862	13.058	4.638
August 23	0.555	30.781	68.664	5.588	74.252	25.193	1.019	1.933	5.047	10.865	3.532	14.397	2.797
" "	0.613	30.896	68.354	5.578	73.932	25.318	1.110	2.031	4.918	10.924	3.239	14.163	3.095
Average	0.584	30.839	68.509	5.583	74.092	25.256	1.065	1.982	4.983	10.895	3.386	14.280	2.946
August 30	0.541	30.856	68.603	5.954	74.557	24.902	1.019	1.813	5.255	9.335	5.608	14.944	1.557
" "	0.641	30.858	68.501	5.349	73.850	25.509	1.142	1.962	5.304	8.680	6.012	14.692	2.400
Average	0.591	30.857	68.552	5.652	74.204	25.206	1.081	1.888	5.280	9.008	5.810	14.818	1.983

TABLE 9. FORMS OF NON-PROTEIN NITROGEN IN ALFALFA CUT AT DIFFERENT PERIODS OF GROWTH.
Results expressed in percent of the water-free substance.

	Total nitrogen in					Nitrogen in the filtrate from the colloidal iron in the form of							
Date 1916	Ether extract.	Water extract.	Residue insoluble in ether and water.	Precipitate produced by the colloidal iron.	Protein nitrogen.	Total.	Ammonia.	Humin.	Free and combined acid-amide.	Free amino.	Combined amino.	Free and combined amino.	Residual soluble nitrogen in the filtrate
June 28	0.052	2.268	3.863	0.103	3.966	2.165	0.275	0.121	0.149	0.622	0.378	1.000	0.611
" "	0.051	2.240	3.891	0.058	3.949	2.183	0.257	0.115	0.147	0.630	0.367	0.997	0.669
Average	0.052	2.254	3.877	0.081	3.952	2.174	0.266	0.118	0.148	0.626	0.373	0.999	0.640
July 5	0.032	1.907	2.869	0.096	2.965	1.811	0.157	0.101	0.206	0.556	0.379	0.935	0.413
" "	0.031	1.911	2.867	0.100	2.967	1.810	0.155	0.133	0.252	0.562	0.372	0.934	0.339
Average	0.032	1.909	2.866	0.098	2.966	1.811	0.156	0.117	0.229	0.559	0.376	0.935	0.376
July 13	0.019	1.405	2.336	0.062	2.398	1.343	0.109	0.077	0.185	0.454	0.235	0.689	0.278
" "	0.021	1.414	2.326	0.076	2.402	1.338	0.108	0.078	0.226	0.455	0.224	0.679	0.300
Average	0.020	1.410	2.331	0.069	2.400	1.341	0.109	0.078	0.206	0.455	0.230	0.684	0.289
July 19	0.016	1.304	1.952	0.143	2.095	1.160	0.088	0.078	0.219	0.441	0.119	0.561	0.215
" "	0.016	1.297	1.959	0.102	2.061	1.195	0.087	0.069	0.171	0.443	0.119	0.562	0.306
Average	0.016	1.301	1.956	0.123	2.078	1.178	0.088	0.074	0.195	0.442	0.119	0.562	0.261
July 26	0.012	0.995	1.735	0.118	1.853	0.878	0.088	0.069	0.155	0.332	0.139	0.471	0.111
" "	0.013	1.001	1.728	0.120	1.848	0.881	0.065	0.069	0.156	0.331	0.138	0.469	0.123
Average	0.013	0.998	1.732	0.119	1.851	0.880	0.077	0.069	0.156	0.332	0.139	0.470	0.117
August 2	0.011	1.006	1.674	0.149	1.823	0.856	0.046	0.055	0.183	0.337	0.106	0.443	0.129
" "	0.011	1.005	1.675	0.148	1.823	0.856	0.046	0.059	0.184	0.321	0.122	0.443	0.124
Average	0.011	1.006	1.675	0.149	1.823	0.856	0.046	0.057	0.184	0.329	0.114	0.443	0.127
August 9	0.010	0.922	1.669	0.155	1.824	0.767	0.031	0.053	0.159	0.337	0.082	0.419	0.105
" "	0.007	0.917	1.676	0.132	1.804	0.785	0.029	0.055	0.163	0.337	0.072	0.409	0.131
Average	0.009	0.920	1.673	0.144	1.814	0.776	0.030	0.054	0.161	0.337	0.077	0.414	0.118
August 16	0.010	0.840	1.824	0.163	1.987	0.677	0.029	0.033	0.131	0.304	0.050	0.354	0.130
" "	0.012	0.845	1.817	0.170	1.987	0.676	0.031	0.051	0.123	0.304	0.049	0.352	0.118
Average	0.011	0.843	1.828	0.167	1.987	0.677	0.030	0.042	0.127	0.304	0.050	0.353	0.124
August 23	0.014	0.794	1.773	0.150	1.923	0.645	0.027	0.050	0.129	0.281	0.094	0.375	0.063
" "	0.015	0.799	1.768	0.145	1.913	0.654	0.029	0.052	0.127	0.282	0.083	0.365	0.080
Average	0.015	0.797	1.771	0.148	1.918	0.650	0.028	0.051	0.128	0.282	0.089	0.370	0.072
August 30	0.014	0.781	1.738	0.150	1.888	0.631	0.026	0.046	0.133	0.237	0.144	0.379	0.047
" "	0.016	0.781	1.735	0.136	1.871	0.645	0.027	0.049	0.136	0.220	0.152	0.372	0.060
Average	0.015	0.781	1.737	0.143	1.879	0.638	0.027	0.048	0.135	0.229	0.148	0.376	0.054

It is evident from a study of the results in Tables 8 and 9 that marked changes take place at the end of the fourth and eighth weeks. These changes are of interest, because they are in accordance with changes noted in the field. For example, it was at the end of the fourth week that the second cutting of the hay took place and at the end of the eighth week that the first decided loss of leaves was reported. In view of these facts, it seemed advisable to class the different stages into three periods and to discuss the results obtained on a basis of periods rather than one of weeks. In the following discussion, it should be borne in mind that Period I consists of the first four weeks, Period II of the next four weeks and Period III of the last two weeks.

ETHER EXTRACT. An examination of Table 8 shows that the percent of the ether soluble nitrogen decreases as the plant matures. The most marked decrease is found in Period I where a change from 0.833 to 0.394 percent takes place, and altho a similar change takes place in Period II, the decrease is only one from 0.497 to 0.394. In Period III, however, an increase occurs, and tho it never reaches that reported at the end of the first week it does, however, approximate that found at the end of the second week. When the results are expressed in percent of the water free plant they show the same tendencies noted above.

WATER EXTRACT. It is obvious from Table 8 that the percents of the water soluble nitrogenous constituents fluctuate in Period I. Expressed as percent of the total nitrogen this fraction first increases from 36 to 39 percent, then decreases to 37 percent and finally rises again to 39 percent. This fluctuation continues during the early part of Period II, but after the second week the amounts decrease regularly and finally become constant in Period III. It is interesting to note that the water soluble nitrogen as expressed in Table 9 exhibits the same tendency to decrease with maturity, but that with exception of the last week in Period II no fluctuation takes place.

PRECIPITATE PRODUCED BY COLLOIDAL IRON. The nitrogen in the precipi-

tate formed by the colloidal iron hydroxide is of interest, because it represents the nitrogen of the water soluble proteins. This form of nitrogen remains fairly constant during the first three weeks of Period I. In the fourth week, however, a decided increase represented by a change from 1.8 to 4.1 percent takes place, and altho this increase continues thruout the next period, it is important to note that the increase during this whole period is but very little more than that which took place during the last week of the preceding period. As has already been mentioned, the total water soluble nitrogen becomes constant in Period III and it is therefore not at all surprising that in this last period the water soluble proteins show a variation of but 0.031 percent. When these proteins are calculated on the basis of the water free feed, the same changes noted above take place, and it will therefore be sufficient to say that a very decided increase takes place some time between the third and fourth weeks, that these constituents continue to increase in Period II and that they remain constant in Period III.

PROTEIN NITROGEN. It is apparent from Table 8 that the protein nitrogen increases as the plant matures. It is interesting to note that these complexes are very constant in Period I, that they increase rapidly in Period II and that they remain constant in Period III. When expressed in percent of the water free feed the situation is just reversed. A rapid decrease from 3.9 to 2.07 percent takes place in Period I and a constant varying only from 1.98 to 1.82 percent is maintained in Period II and III.

TOTAL NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON. Taken as a whole, the non-protein nitrogen as expressed in Table 8 decreases from 35 to 25 percent. It is important to note, however, that this decrease does not proceed at the same rate in the different periods. In Period I there is even an increase at the end of the second week, but this is immediately followed by a drop to the original level which is maintained for the rest of the period. Some time between the first two periods another sudden drop occurs. Thus a decrease takes place

in every stage, but nevertheless attention should be called to the fact that these constituents have almost approached a constant by the end of the eighth week. Comparing the results in Table 9 with those discussed above it is evident that the same changes have taken place when the data compiled in both is taken as a whole. In other words, the non-protein nitrogen when calculated on the basis of the water free feed decreases from 2.17 to 0.638 percent. A more detailed study of the results obtained show, however, that there are no fluctuation in the percent of these constituents as they are expressed in Table 9.

FREE AMMONIA NITROGEN IN THE WATER EXTRACT. The decrease of the free ammonia in the water extract is unquestionably the best example of a loss of the simpler nitrogenous constituents with maturity, because without exception a decrease in these takes place every week. This holds whether the results are expressed in percent of the total nitrogen or calculated on the basis of the water free substance.

HUMIN NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON. Some if not all of the humin nitrogen owes its origin to the destructive action of hydrochloric acid on the amino-acids, and it is of value to note that while the humin nitrogen is almost constant when expressed in percent of the total nitrogen, it decreases with the amino-acids when expressed in percent of the water free substance. This indicates that the actual amount of humin nitrogen formed depends upon the total amino-acid linkings present.

FREE AND COMBINED ACID-AMIDE NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON. Concerning the free and combined acid-amide nitrogen, Table 8 shows that taken as a whole these constituents show a maximum increase from 2.390 to 6.778 percent when expressed in percent of the total nitrogen and an analogous one of from 0.148 to 0.229 percent when considered as a part of the water free substance. It is important to note, however, that whereas this maximum is reached at the end of the second week in Table 9 it is not attained until the

third week in Table 8. A further consideration of Table 8 reveals a progressive increase during the first period. This increase proceeds through the second week of Period II, and while a disappearance of amides takes place in the last weeks of this period they are again built up in Period III.

FREE AMINO-ACID NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON. The free amino-acids when expressed in percent of the total nitrogen gradually increase during the early stages of growth. This is pointed out in Period I in Table 8. The tendency in the later stages is for this form of nitrogen to decrease and with exception of the third week of Period II this decrease takes place week by week. It is clear from Table VII that when these linkings are expressed in percent of the water free substance a decrease takes place every week.

COMBINED AMINO-ACID NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON. The demarcation between Periods I, II, and III is best emphasized by a study of the combined amino-acid nitrogen as expressed in Table 8. In the first place it is apparent that a disappearance of peptide nitrogen takes place in the first eight weeks, and further that these forms are built up again in Period III. In the second place, it is important to see that a sudden break takes place some time between Periods I and II for whereas a loss from 6.0 percent to 3.6 percent is found in Period I this is immediately followed by an increase in the first week of Period II. After this time, the changes proceed along lines analogous to those noted above in Period I. Similar changes occur when the combined amino nitrogen is calculated on the basis of the water free substance.

FREE AND COMBINED AMINO-ACID NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON. It is evident from Table 8 that the free and combined amino-acids are being built up during the first half of Period I, and that they are gradually disappearing during the remaining stages. Table 9 reveals similar changes, but it is interesting to note that the decrease commences with the first week and is not interrupted until the plant enters Period III.

RESIDUAL SOLUBLE NITROGEN IN THE FILTRATE FROM THE COLLOIDAL IRON.

Tables 8 and 9 show that the unknown constituents decrease as the plant matures, and, altho there is a slight fluctuation in Period II, there is nevertheless a a loss from period to period. Taken as a whole, these forms decrease from 10 to 2 percent when expressed in percent of the total nitrogen and from 0.64 to 0.05 percent when calculated on the water free basis.

INTERPRETATION OF RESULTS OBTAINED.

In interpreting the results obtained no attempt will be made to set up any theory of protein synthesis, but an effort will be made to show how the results confirm those of other investigators, and what relationship exists between the non-protein and protein nitrogen. The interpretation of the data will be confined to a discussion of the first eight samples of the hay, because those of the last two weeks are not representative of the whole plant. Furthermore, it will be limited to the results expressed in percent of the water free feed.

TABLE 10. WEEKLY INCREASES AND DECREASES OF PROTEIN AND NON-PROTEIN NITROGEN.

Expressed in percent of the total nitrogen.

	Protein nitrogen.	Free and combined amino-acid nitrogen.	Ammonia nitrogen.	Acid-amide nitrogen.	Humin nitrogen.	Residual nitrogen.	Difference in gain and loss.
2nd week	-2.210	+3.285	-1.042	+2.114	0.157	-1.826	0.157
3rd week	+2.140	-1.246	-0.358	+0.511	+0.320	-0.998	0.369
4th week	+0.061	-0.027	-0.235	+0.923	+0.141	-0.090	0.227
5th week	+3.670	-0.016	-0.152	-0.290	+0.271	-3.361	0.122
6th week	+0.218	-0.683	-0.800	+1.112	-0.256	+0.374	0.035
7th week	+3.322	-0.549	-0.522	-0.586	-0.159	-0.403	1.003
8th week	+4.245	-2.864	-0.088	-1.452	-0.477	-0.386	1.022
Total	+11.446	-3.100	-3.197	+2.328	-0.287	-5.690	1.500

Table 10 shows how these protein and non-protein nitrogenous constituents increased and decreased during the first eight weeks. The last column in this table is of interest, because it shows that the difference between the total loss and gain is very small, and that all of the constituents must be considered

in attempting to explain these losses and gains. Turning back to Table 8, it is clear that with the exception of the free and combined acid-amides, the constituents of the non-protein fraction decreased as the total protein increased, and while there is nothing conclusive in such evidence it is likely from the additional evidence in Table 8 that the simpler constituents were used up in the formation of the complex protein molecule.

It has been pointed out that ammonium salts, nitrates, and nitrites are intimately connected with protein synthesis. This point has been in part substantiated in this investigation. The data shows that ammonia or ammonium salts are involved in the increase of proteins, because such increase was accompanied by a loss of ammonia nitrogen. No direct determination of nitrites or nitrates was made, but it is obvious that these would appear in the residual non-protein nitrogen fraction, and a glance at the data shows that this residual nitrogen decreased in a manner similar to that in the ammonia.

Mention was also made of the fact that amino-acids would increase at the expense of nitrates. Table 10 shows that an increase of amino-acid linkings took place during the second week, and while some of these probably originated from the decomposition of the proteins this will not explain all of the increase. The only other possibility is that some of the residual soluble nitrogen was utilized for amino-acid synthesis, and it is likely that the nitrates present in that fraction were responsible for the changes noted. The free and combined acid-amide nitrogen also increased during this second week and in explaining such an increase the logical conclusion is that it was made at the expense of the free ammonia. But the table shows clearly that the increase of amide nitrogen was greater than the decrease of the ammonia nitrogen, and it would seem then that, while the decomposition of the proteins may account for some of the increase in the amino-acids, a similar assumption might be made with regards to the formation of amide nitrogen.

During the third week, there was an actual gain of 2.04 percent of protein nitrogen and 0.511 percent of free and the combined acid-amide nitrogen. The simpler constituents decreased, but it should be seen that the increase could not be wholly explained by a loss of ammonia and amino-acid nitrogen, but must include some of the residual non-protein nitrogen. Further, it is plain that the increase of the amide nitrogen cannot be all due to loss in ammonia nitrogen, and it is therefore possible that some of the amino-acids were deaminized or some of the residual nitrogen utilized.

In the fourth week the amide nitrogen increased quicker than the proteins. As in the preceding week, the increase of amide nitrogen can not all be due to combination of ammonia with acids, and it is possible that some of the residual nitrogen was changed into ammonia and used for the synthesis of amides. But this again will not explain all of the increase, and one must look for a deaminization of amino-acids to explain the rest of the amide formation.

Table 10 shows that a marked rise in protein nitrogen took place in the fifth week, and further that all of the constituents of the non-protein fraction decreased. It is important to note that the sum of the amino-acid nitrogen, the free ammonia nitrogen, and the amide nitrogen will not account for all of the protein nitrogen increase. It is likely that some of the proteins arose from the residual nitrogen. In a similar manner, the sum of the amino-acid nitrogen, the free amino-acid nitrogen, and the residual nitrogen will not account for the protein formation. This shows that the amides might also be responsible for some protein synthesis.

The changes in the sixth week are, to some extent, in accordance with those noted in the fourth week. It is shown again that, whereas the loss in ammonia nitrogen will account for some of the rise of the amide fraction, it will not account for all of it, and further that it is quite possible that the additional increase may be due to deaminization of the amino-acids. Another point,

which should be mentioned, is that the residual soluble nitrogen increased. This may be due to the fact that an error was made in the taking of the samples, or that some of the known constituents were broken down.

The table shows a progressive increase of protein nitrogen and a similar decrease of all of the constituents of the non-protein fraction in the last two weeks. It is evident that all of the constituents must be taken into consideration to explain the increase of the proteins in the seventh week. In the last week, it is possible to exclude the ammonia and the residual nitrogen, but some of the amino-acids and some of the amides must be included.

The figures at the bottom of Table 10 represent the total increases and the total decreases. The sum of all of the increases is 13.774 percent as compared with 12.274 percent for the decreases. It is evident that there must be some close relation between the fractions that disappear and those that are built up. That all constituents are probably involved follows from the comparison of decreases and increases in the 5th, 7th and 8th weeks; that the ammonia nitrogen will not account for all of the increase of amide nitrogen is apparent from a consideration of every week where such an increase takes place; that the amino-acids may be responsible for some of the amide formation is evident from that part of the table relating to the 2nd, 3rd, 4th and 6th weeks; that the residual nitrogen may be connected with protein synthesis is apparent from the 3rd, 5th and 7th weeks; and that the amino-acids are intimately related to protein synthesis is shown in the 3rd, 7th and 8th weeks.

The summary made in the preceding paragraph, while it is not based upon absolute experimental evidence, is, nevertheless, in accordance with our knowledge of the structure of the protein molecule. It is well agreed that the amino-acids pre-exist in the protein molecule, and that protein like complexes can be synthesized from amino-acids. It is, therefore, logical to expect that in nature the proteins are synthesized from the amino-acids. Furthermore, it has been shown

that the hydrolysis of proteins gives rise to amide linkings which probably originate from the amides of the dicarboxylic amino-acids, aspartic and glutamic acid. In experiments of this nature, it is therefore natural to expect a decrease of amino nitrogen with an increase of protein nitrogen, and further a decrease of amide nitrogen with a similar increase in protein nitrogen. While it is true that the increase in protein nitrogen was not always accompanied by a decrease of amide nitrogen, it is, nevertheless, true that evidence has been obtained which cannot be explained in any other way than that the amides were in some way connected with protein synthesis. Finally the changes of the ammonia and residual nitrogen were what one would expect if the work on the relation of these to protein synthesis were true.

PART IV

CONCLUSIONS

1. A method has been devised by which a fairly good picture of the non-protein nitrogenous constituents of feedingstuffs can be obtained.

2. The non-protein nitrogenous constituents, while they are quite constant in the same type of feedingstuff, vary with the different types of feedingstuffs

3. The non-protein nitrogen fraction of the total nitrogen decreases with the maturity of alfalfa hay, and, as a result of this, the protein fraction increases.

4. All of the constituents of the non-protein fraction of alfalfa hay are intimately connected with protein synthesis.

BIBLIOGRAPHY

1. Jour. prak. Chem., 5 (ns),215,(1872).
2. Jour. Landw., 28, 103, (1880).
3. Zeit. fur Anal. Chem., 39, 545, (1900).
4. Jour. Biol. Chem.,11, 527, (1912).
5. Jour. Biol. Chem.,26, 491, (1916).
6. Jour. Biol. Chem.,21, 61, (1915).
7. Jour. Biol. Chem.,34, 97, (1918).
8. Jour. Biol. Chem., 25, 210, (1916).
9. Unpublished work of Grindley and Slater.
10. Jour. Biol. Chem., 22, 477,(1915).
11. Jour. Biol. Chem., 20, 175, (1915).
12. Jour. Biol. Chem., 23, 381, (1915).
13. Jour. Biol. Chem., 10, 15, (1911).
14. Ann. Inst. Pasteur. 14, 26.
15. Ber. bot. Gesells., 15, 5361, (1897).
16. The following references apply only to alfalfa hay:

    Colorado Agr. Exp. Sta. Bul. 8, 9.

    Colorado Agr. Exp. Sta. Bul. 35, 9.

    Colorado Agr. Exp. Sta. Bul. 39, 3.

    Kentucky Agr. Exp. Sta. Report 1888, p. 18.

    Minnesota Agr. Exp. Sta. Bul. 80, 149.

    Nevada Agr. Exp. Sta. Bul. 63, 37.

    New Jersey Agr. Exp. Sta. Bul. 148, 12.
17. Utah Exp. Sta. Bul. 44, 13.

    Utah Exp. Sta. Bul. 48, 75.

    Utah Exp. Sta. Bul. 58, 90.

    Utah Exp. Sta. Bul. 61, 169.

18. Landw. Jahrb., 25, 621, (1906).

19. Bot. Zeitung., 36, 801, (1878).

20. Landw. Jahrb., 2, 243, (1883).

21. Ber. deut. bot. Gesells., 23, 126, (1905).

Beit. bot. Centr., 27, 63, (1911).

22. Ber. deut. bot. Gesells., 44, 1009, (1911).

23. Ann. jard. bot. Buitenzarg., 13, 1, (1895).

Ann. jard. bot. Buitenzarg., 19, 86, (1904).